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Untargeted metabolomic reveals changes in serum metabolism in peanut allergic mice treated by raw and roasted peanuts

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ABSTRACT

Peanut allergy (PA), which affects an increasing proportion of people, is gaining increasing interests. Clarification of the mechanism underlying PA may aid in the identification of novel biomarkers and strategies for clinical intervention in allergic patients. In this study, raw peanut protein (Raw), roasted peanut protein (Roast), high-molecular-weight protein aggregation from Roast (OH), and the remaining components excluding OH from roasted peanut (OL) were administrated *via* intraperitoneal injection to challenge BALB/c mice. The allergic response was assessed, and the levels of peanut-specific immunoglobulin E, mouse mast cell protease-I, and T-lymphocyte subsets and secreted cytokines were detected. Then, serum untargeted metabolomics was investigated. Results show that Raw induced robust anaphylaxis in mice and up- or down-regulated 269 serum metabolites that mainly affected amino acid metabolism, including tryptophan metabolism, and induced disruption of tricarboxylic acid (TCA) cycle pathway in serum. Roast, OH and OL induced mild anaphylaxis in mice and mainly affected the glycerophospholipid metabolism in serum. Compared with Tris, OH upregulated the glycerophospholipid metabolism pathway in mouse serum, whereas Roast and OL showed the reversed effect. Mice in the Roast, OL, and OH groups all showed upregulated glycerophospholipid metabolism in serum compared with those in the Raw group. Meanwhile, different components of roasted peanut caused various changes in pathways when compared with each other. Compared with mice in the Roast group, those in the OL group displayed downregulated tryptophan and tyrosine metabolism, and those in the OH group showed upregulated glycerophospholipid metabolism and downregulated TCA cycle. The differences in serum metabolic profiles in mice implied that the processing form of allergen that patients are sensitized to and the severity of allergy should be considered in the analysis of serum biomarkers.

1. Introduction

Peanut allergy (PA) is an important public health problem worldwide[1-2], and patients with PA suffer from an evident decreased quality of life[3-4]; they may experience itching, swelling, vomiting, hives, arrhythmia, and other symptoms with minimal exposure to peanut allergen and may die due to severe allergic reactions[5]. PA is typically an immunoglobulin E (IgE)-mediated T helper type 2 (Th2)-polarized immune response[6]. After antigens have been taken up by dendritic cells, B cells or other antigen-presenting cells, are presented to cognate naive T cells and acquire a Th2 cell phenotype in the presence of interleukin (IL)-4 or IL-13. Then, Th2 cells engage cognate B cells and secrete IL-4 and IL-13 to induce the secretion of antigen-specific IgE by B cells[7].

The most effective treatment for PA is the strict avoidance of peanut exposure. To reduce the allergic reactions resulting from accidental ingestion, scholars have developed innovative treatments, including oral[8], sublingual[9], and epicutaneous immunotherapy[10].

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Although several immunotherapies have been proposed and optimized for PA, their side effects and safety remain a concern[11]. Such outcomes maybe related to the unclear understanding the immune mechanisms underlying specific allergic reactions. Currently, several studies have performed on untargeted metabolomic profiling to identify candidate disease biomarkers for food allergy patients[12-13]. A total of 8 well-known allergic foods and other potentially allergic consumables can be found around the world[14]. Among allergies induced by different foods, the eliciting doses (ED), symptoms, and the ability to spontaneously recover with the increase in age vary. Thus, studies on candidate disease biomarkers for food allergic patients should be conducted separately for each type of food. PA commonly shows a low ED[15] and a high fatality rate[4], which illustrates the urgent need for in-depth clarification of the immune mechanisms underlying PA. Therefore, discovering the effect of PA on serum metabolism may be favorable for promoting the development of PA treatments.

PA is a complex disease related to genetic and environmental features and implicates thermal processing[16]. Above 80% of peanuts are used for roasting[17], direct consumption, or use as food ingredients to prepare various snack foods, such as peanut butter, candies, and milk[18-19]. In addition, the structure and allergenicity of peanut protein is more susceptible to roasting rather than other cooking methods (boil or steam). Therefore, the research on the mechanism behind roasted peanut allergenicity may be closely related to clinical practice. In terms of structure, roasting changes the structure of allergen monomers[20-21], especially, the conformational structure plays a critical role in changes in allergenicity[21]; high-molecular-weight protein aggregation (OH) occurred in roasted peanut and shows an evident IgE recognition capability[22]. In term of sensitization mechanism, roasted allergen increases the secretion of reactive oxygen species of intestinal epithelial cells[23]. However, these studies are still insufficient to explain how roasting induces changes in the allergenicity of peanut protein. Insights into the effect of roasted peanut on the body's metabolism contribute to promoting the exploration of immunological mechanisms. In addition, with OH as the evident protein product induced by roasting peanut, assessment of the anaphylactic capacity and analysis of serum metabolism induced by OH may provide new insights into the field of immunological characterization of protein aggregation induced by processing.

In this work, we investigated the effect of PA development on the composition of serum untargeted metabolomics in mice and assessed the changes in anaphylactic potency and serum metabolomics in mice after the challenge using the total protein and protein components from roasted peanut. In detail, peanut-sensitive mice were obtained *via* multiple gavage administrations of raw peanut. The sensitive mice were challenged through intraperitoneal injections of Tris, raw peanut protein (Raw), roasted peanut protein (Roast), OH, and OL (the remaining components excluding OH from roasted peanut) to assess the anaphylactic capacities of samples. Spleen and mesenteric lymph nodes (MLN) were collected for *in vitro* immune analysis. Blood was collected for the detection of specific antibodies and untargeted metabolome. This study provides novel insights into the field of potential metabolomic markers for PA.

2. Material and method

2.1 Sample preparation

The samples were prepared in accordance with the method previously described by Zhang et al.[21]. Sun-dried fresh peanuts (raw peanut, variety: Huayu 16) were roasted at 170 ℃ for 12 min and then cooled, skinned, and finely milled. The obtained powder was defatted with acetone, and the defatted peanut powder (DPP) was stored at –20 ℃ until use. The DPP of raw peanut was prepared in the same manner.

Raw and Roast were extracted by mixing the DPP with Tris-HCl buffer (pH 8.0)[24], and the concentration of total peanut protein in samples was determined *via* the Bradford protein assay[25].

Protein components were separated from Roast through anion exchange chromatography[21]. The total protein from roasted peanut was loaded onto a diethylaminoethyl (DEAE)-sepharose fast flow resin column. The column was equilibrated with 50 mmol/L Tris-HCl (pH 7.2), and the proteins were eluted successively with 600 mL 0–0.3 mmol/L NaCl, 100 mL 0.3 mmol/L NaCl, 100 mL 1.2 mmol/L NaCl, and 100 mL 0.5 mmol/L NaOH in 50 mmol/L Tris-HCl at the rate of 1.5 mL/min. Different fractions were collected and identified using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions comprising allergen monomers were mixed and concentrated using ultrafiltration tubes at a cut-off value of 3.5 kDa, and those belonging to OH were concentrated using ultrafiltration tubes with a cut-off value of 100 kDa. The final samples were identified *via* SDS-PAGE.

2.2 Animals

BALB/c mice (specific pathogen-free grade, female, aged 4–5 weeks, and weighed of 16–17 g) were obtained from Hunan Slake Jingda Laboratory Animal Co., Ltd. (Hunan, China). The mice were housed in a specific pathogen-free facility, with a controlled temperature of (25 ± 1) ℃, around 50% humidity, and a meticulously regulated 12 h light/dark cycle. All mice were provided with a peanut-free diet and sterile water. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Jiangxi Chinese Medical University (Approval No.: TEMPOR20230089).

2.3 Peanut sensitization and challenge in mice

Peanut-sensitive mice were prepared in accordance with a previously described method[26]. The mice were randomly divided into 5 groups (n = 6). On days (0, 1, 2, 7, 14, 21, and 29), the mice were sensitized by intragastric gavage (i.g.) with 5 mg raw peanut extract mixed with 15 μg cholera toxin B subunite (CTB, Absin, Shanghai, China) in 200 μL Tris-HCl (pH 8.2). On the 37th day, the mice in one group were injected intraperitoneally (i.p.) with 200 μL Tris-HCl (pH 8.2) (as Tris group), and those in another four groups were i.p. challenged with 800 μg Raw, Roast, OH, and OL in 200 μL volume. On the 39th day, a secondary challenge was administered to the mice with a dose of 1 000 μg proteins. The mice were observed for 60 min after the challenge, and the allergic symptoms were scored in accordance with the scoring rules in Table S1. The following day,

blood was gathered in serum procoagulation tubes and left to stand for 2 h for centrifugation. Then, the serum in the supernatant was collected and stored at –80 ℃.

2.4 Detection of peanut protein-specific IgE (PP-sIgE) and mouse mast cell protease-I (mMCP-1) in serum

Serum PP-sIgE was detected *via* an indirect enzyme-linked immunosorbent assay (ELISA)[27]. Briefly, the 96-well plates were coated with raw peanut protein at 4 ℃ overnight. The plates were blocked with 5% defatted milk powder in phosphate-buffered saline (PBS) at 37 ℃ for 2 h and reacted with the sera at 37 ℃ for 1 h. The sera were diluted by factors of 10 for detection. Mouse IgE was recognized using the goat anti-mouse IgE-biotin antibody at 37 ℃ for 1 h and subsequently reacted with horseradish peroxidase-labeled avidin. Finally, tetramethylbenzidine solution was added, the reaction was stopped with sulfuric acid (2 mol/L), and the absorbance was measured at 450 nm. The serum mMCP-1 level was quantified using an ELISA kit (Invitrogen, Carlsbad, CA, United States) following the manufacturer's instructions.

2.5 Lymphocyte analysis of spleen and MLN

2.5.1 Isolation and in vitro restimulation of lymphocytes

Mouse spleen was isolated aseptically from mice and ground with Roswell Park Memorial Institute (RPMI) 1640 medium. After filtering the ground spleen through a nylon membrane cell strainer, the cells were treated *via* erythrocyte lysis for 5 min, and the mixture was washed and suspended in a complete culture medium (RPMI-1640 containing 15% fetal bovine serum (FBS) and 1% penicillin-streptomycin) as mononuclear splenocytes. Splenocytes (5×10^6/mL) were seeded into 24-well culture plates, restimulated with Raw, Roast, OL, OH, and Tris buffer, and incubated for 72 h at 37 ℃ with 5% CO_2. After incubation, the supernatant was collected, and the levels of IL-4, IL-5, IL-13, and interferon (IFN)-γ were detected using commercially available ELISA kits (Invitrogen, Carlsbad, CA, USA). MLN were isolated and treated in the same manner as splenocytes except that the exclusion of the lysis step.

2.5.2 Detection of T-lymphocyte subsets in splenocytes and MLN

Th1/Th2 cells were detected using a flow cytometer. Mononuclear splenocytes were determined as above described. A total of 2.5×10^6 of splenocytes were placed in 96-well plates, and nonspecific binding to Fc receptors was blocked with anti-CD16/32. Then, the cells were incubated with anti-mouse CD4, T-bet, and GATA3 antibodies. Finally, cells were resuspended in 300 μL flow buffer (PBS containing 15% FBS). Th1 cells were identified as $T\text{-}bet^+CD4^+$cells and Th2 cells as $GATA3^+CD4^+$cells.

2.6 Untargeted metabolomics analysis of mouse serum.

2.6.1 Metabolite extraction

Metabolite extraction was performed in accordance with the work of Jiang et al.[28]; 300 μL extraction solution (methanol: acetonitrile = 1:1, supplemented with 0.02 mg/mL *L*-2-chlorophenylalanine) was added to 100 μL serum samples. The metabolites were extracted ultrasonically for 30 min at 5 °C and 40 kHz after vortexing for 30 s. The extract was stored at –20 ℃ for 30 min and centrifuged for 15 min (13 000 r/min, 4 °C), and the supernatant was dried with nitrogen. The pellet was redissolved using the reconstitution solution (acetonitrile:water = 1:1) for the secondary extraction based on the above condition. After centrifugation, the obtained cleared supernatant was used for liquid chromatography-mass spectrometry (LC-MS) detection. As a part of system conditioning and quality control process, a pooled quality control sample was prepared by mixing 20 μL of all samples.

2.6.2 Chromatographic conditions

A total of 3 μL samples were separated using an ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm i.d., 1.8 μm; Waters, Milford, USA). The injection volume was 3 μL, and the column temperature was 40 °C.

2.6.3 MS conditions

The mass spectrometric data were collected using a UHPLC-Exploris240 mass spectrometer (Thermo Scientific) equipped with an electrospray ionization source operating in either positive or negative ion mode. The optimal conditions were as follows: heater temperature, 350 °C; capillary temperature, 320 °C; ion-spray voltage floating, –3 000 and 3 400 V in negative and positive modes, respectively. The detection was carried out over a mass range of *m/z* 70–1 050.

Data preprocessing and annotation after MS detection. The RAW data of LC-MS were processed using Progenesis QI v3.0 (Waters Co., Milford, USA) software, which includes sample information, metabolite name, and mass spectral response intensity. The metabolites were searched and identified using HMDB and Meltin Database. The data after the database search were uploaded to Majorbio cloud platform (https://cloud.majorbio.com) for data analysis.

2.7 Statistical analysis

Statistical analysis was conducted *via* Student's unpaired two-tailed *t*-tests or Mann-Whitney U-test using GraphPad Prism 8.0 software. The results are expressed as the mean ± standard error of the mean (SEM), and differences in the mean values were considered significant at $P < 0.05$. Differential metabolite analysis was performed on Majorbio cloud platform. R package ropls (Version 1.6.2) was used to perform orthogonal least partial squares discriminant analysis (OPLS-DA). Student's *t*-test and fold difference analysis were also conducted. Significantly different metabolites were selected based on the variable importance in the projection (VIP) obtained using the OPLS-DA model and the *P*-value of student's *t*-test, and metabolites with VIP > 1 and $P < 0.05$ were considered significantly different.

3. Results

3.1 Separation of protein components from Roast

Aggregated proteins exhibit an increased average net charge because they contain more N-terminal and C-terminal than

monomers; the DEAE-Sepharose column has consistently been used for charge-based protein separation[29]. Therefore, anion exchange chromatography was performed to separate OL and OH from Roast. Fig. 1 shows the protein patterns obtained for Raw, Roast, OL, and OH. Roast displayed significantly dark bands with molecular weights over 130 kDa at the top of the separating gel and over the stacking gel. In the OL lane, the bands almost exclusively presented allergen monomers, with bands over the stacking gel disappearing and minimal shallow bands appearing over 130 kDa. Evidently, the band intensities of allergen monomers Ara h 2 and Ara h 6 increased (around 15 kDa). The OH bands mainly consisted of proteins over 130 kDa and a small amount of proteins under 100 kDa.

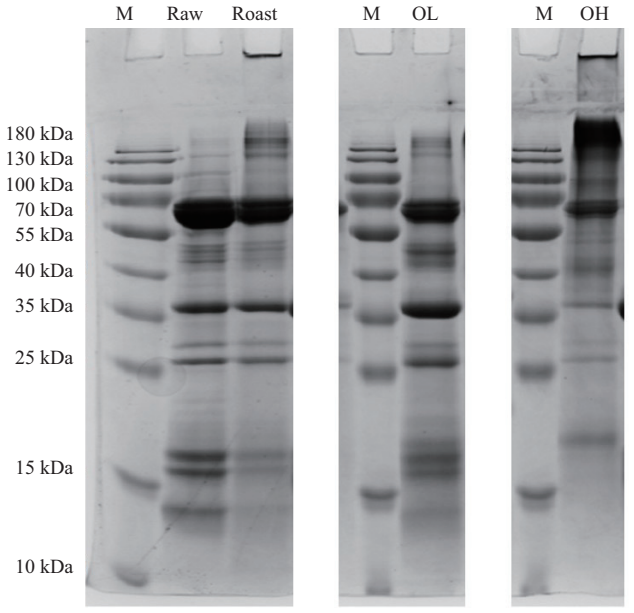

Fig. 1 The protein patterns of Raw, Roast, OL and OH. M: protein ladder.

3.2 Assessment of anaphylactic reaction of mice after challenges

To construct a robust peanut allergic mouse model, we sensitized female BALB/c mice i.g. with raw peanut/CTB and i.p. challenged them twice using Raw or Tris (Fig. 2A). To assess the anaphylactic reaction of mice after repeat challenges, we used the anaphylactic scores and dropped levels in body temperatures as correlative parameters for anaphylaxis[30]. Compared with the Tris group mice, those in the Raw group exhibited marked anaphylactic scores and drops in core body temperature (Figs. 2B and 2C), which indicate evident allergic reactions. We further evaluated the mouse serum level of PP-sIgE. Results show that the level of PP-sIgE in the Raw group mice increased significantly compared with that in the Tris group (Fig. 2D), with raw peanut protein challenges significantly increasing the PP-sIgE level. To analyze the mast cell activation under immunologic stress, we quantified serum mMCP-1 level after the challenges. As shown in Fig. 2E, the Raw group mice exhibited evidently increased serum level of mMCP-1 compared with the Tris group mice.

To gain insights into the changes in the allergenicity of protein components from roasted peanut, we i.p. challenged the sensitized BALB/c mice twice using different protein components from roasted peanut (Roast, OL, and OH groups in Fig. 2A). Compared with the Tris group mice, those in Roast, OL, and OH groups did not exhibit an evident scratching behavior and drop in body temperature, those in the OL group only showed significantly increased level of PP-sIgE, and those in the OH group presented increased levels of PP-sIgE and mMCP-1. Compared with mice in the Raw group, those in the Roast group showed a significantly lower drop level in the core body temperature, those in the OL group exhibited a significant

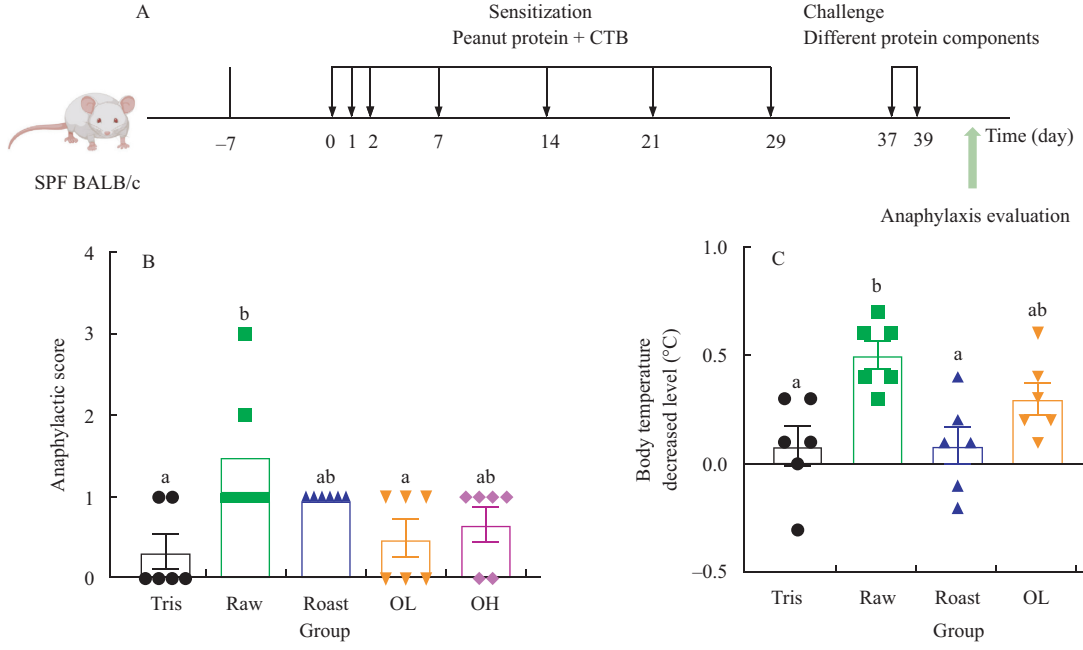

Fig. 2 Anaphylaxis of mice and levels of peanut specific IgE, and mMCP-1 in serum of mice. (A) Experimental design for inducing PA. (B) Anaphylactic score, (C) decreased levels of body temperatures of mice, levels of peanut specific. (D) IgE, and (E) mMCP-1 in serum of mice after the i.p. challenge with Tris buffer (Tris), the Raw, Roast, OL, and OH ($n = 6$). Different letters indicate significant differences ($P < 0.05$).

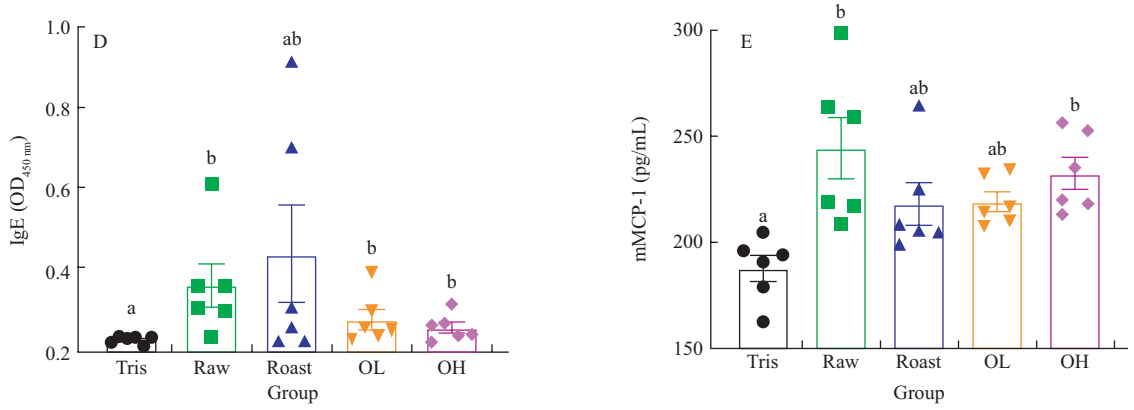

Fig. 2 (*Continued*)

decrease in anaphylactic score, and those in the OH group presented no significant changes. In addition, anaphylaxis and specific antibodies and mMCP-1 in the serum from the Roast, OL, and OH groups showed no remarkable difference.

3.3 *Differentiation of T cells in spleen and MLN*

To determine the specific differentiation of T-cell subsets in mice, we analyzed the fresh splenocytes and MLNs by flow cytometry (Fig. 3). Flow cytometric analysis of splenocytes revealed that the expansion of $CD4^+GATA3^+$ Th2 cells were distinctly increased in the Raw group mice compared with those in the Tris group, and the expansion of $CD4^+T\text{-}bet^+$ Th1 cells did not change significantly. Compared with the Raw group, the expansion of $CD4^+GATA3^+$ Th2 cells in mice in the Roast group significantly decreased, with a slight increase in the percentage of $CD4^+T\text{-}bet^+$ Th1 cells. For splenocytes in mice of the OL and OH groups, the percentages of $CD4^+T\text{-}bet^+$ Th1 and $CD4^+GATA3^+$ Th2 cells were both significantly decreased compared with those in the Raw group, and those of $CD4^+T\text{-}bet^+$ Th1 cells were evidently lower than those in the Roast group.

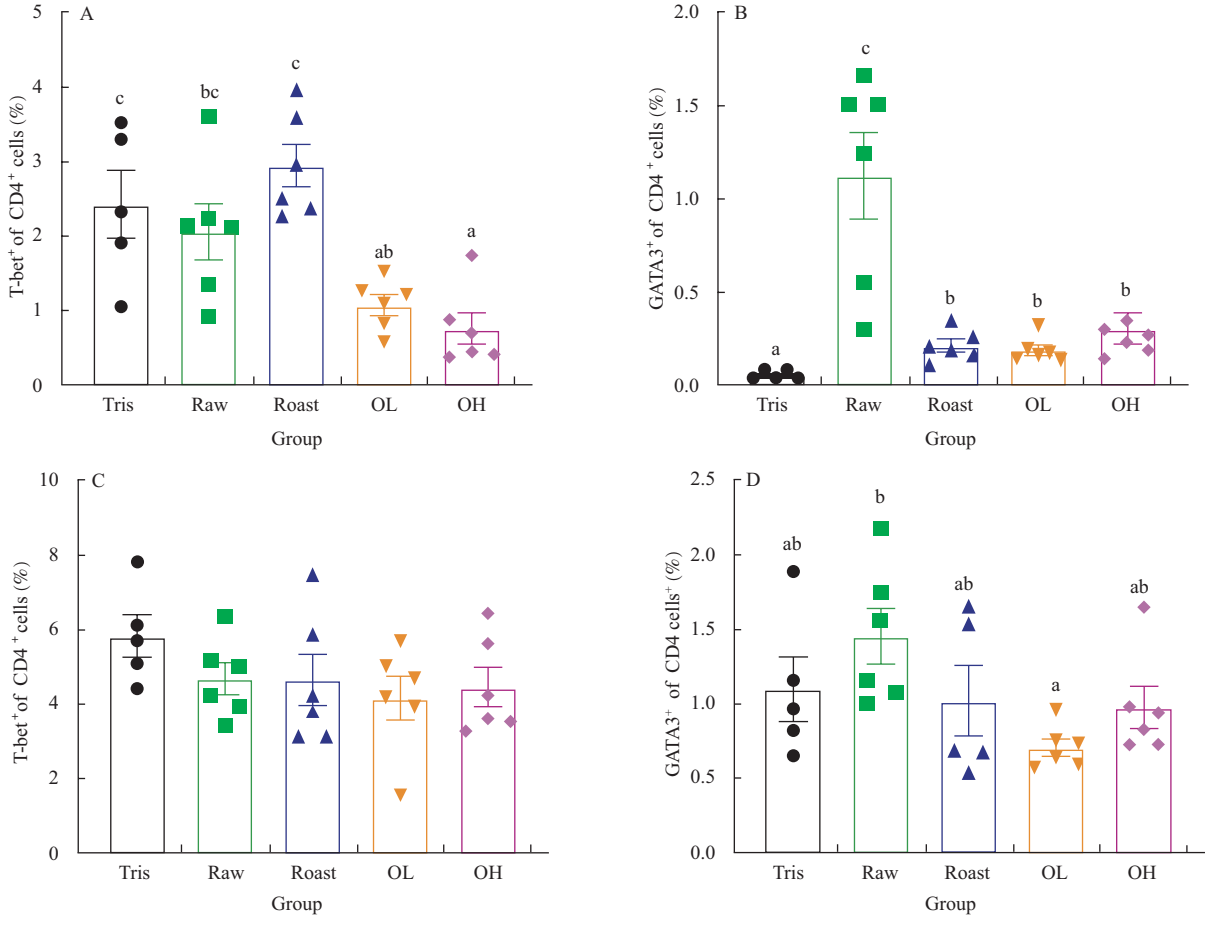

Fig. 3 Flow cytometric analysis of Th cells in Spleen and MLN in mice. Th1 cells were defined as $T\text{-}bet^+$ of $CD4^+$ cells and Th2 cells as $GATA3^+$ of $CD4^+$ cells. Percentages of (A) splenic Th1 cells, (B) splenic Th2 cells, (C) MLN Th1 cells, and (D) MLN Th2 cells. Different letters indicate significant differences ($P < 0.05$).

Changes in the differentiation of T cells in MLN varied from those in spleen. Only the OL group exhibited a significant decrease in the level of $CD4^+GATA3^+$ Th2 cell differentiation compared with the Raw group. No significant changes in T cell differentiation were observed among the other groups.

3.4 Cytokine production after in-vitro restimulation with proteins

Spleen, a secondary lymphoid organ, is a site where deleterious immune responses can be regulated[31]; in this study, T cell differentiation was considerably changed after the mice were challenged with proteins. Consistent with the results of $CD4^+$ T cell polarization in mice, the cytokine levels typically associated with Th2 cells (Th2-specific cytokines, including IL-4, IL-5 and IL-13) were significantly elevated in the supernatant of splenocytes in the Raw group, and the level of INF-γ (associated with Th1 cells) was evidently decreased compared with that in the Tris group (Fig. 4).

For the Roast group, the change tendency of the released levels of cytokines were consistent with that in the Raw group compared with that in the Tris group. Meanwhile, compared with those in the Raw group, the levels of Th2-specific cytokines in the supernatant of splenocytes in the Roast group exhibited remarkably lower levels (Figs. 4A-C), and that of Th1-associated cytokine showed no marked change (Fig. 4D).

As for the OL and OH groups, the levels of Th2-specific cytokines in the supernatant of splenocytes were significantly lower, and that of IFN-γ was significantly higher than those in the Raw group (Fig. 4). Compared with the Roast group, the OL and OH groups exhibited significant decreases in the released level of IL-4 (Fig. 4A) and an evident increase in the released level of IFN-γ (Fig. 4D). We also observed differences between the OL and OH groups. The level of IFN-γ (Fig. 4D) in the supernatant of splenocytes in the OH group was significantly higher than that in the OL group.

3.5 Alteration of serum metabolite profiles in peanut-allergic mice

To analyze the alteration of serum metabolite profiles in peanut-allergic mice, we mapped the global metabolomic profiles of the mice in the Raw and Tris groups using the technique of untargeted metabolomics. The result of OPLS-DA model showed significant inconsistencies between the serum metabolic profiles of mice in the Raw and Tris groups (Fig. 5A). As shown in Fig. 5B, 269 different metabolites with $P < 0.05$ and VIP > 1, which mainly included amino acids, peptides, and analogues, fatty acids and conjugates, carbohydrates and carbohydrate conjugates, and glycerophosphoethanolamines, were obtained. The levels of 65 metabolites, including two kinds of phosphatidylcholine, isocitrate, and mesaconic acid, were upregulated, and those of 204 metabolites, including cytidine diphosphate-diacylglycerol, *N*-α-acetyl-*L*-arginine, and *N*-methyl-*L*-glutamic acid, were decreased significantly in the Raw group.

Differential metabolites between Raw and Tris groups were summarized and mapped into their biochemical pathways through metabolic enrichment and pathway analysis based on database search

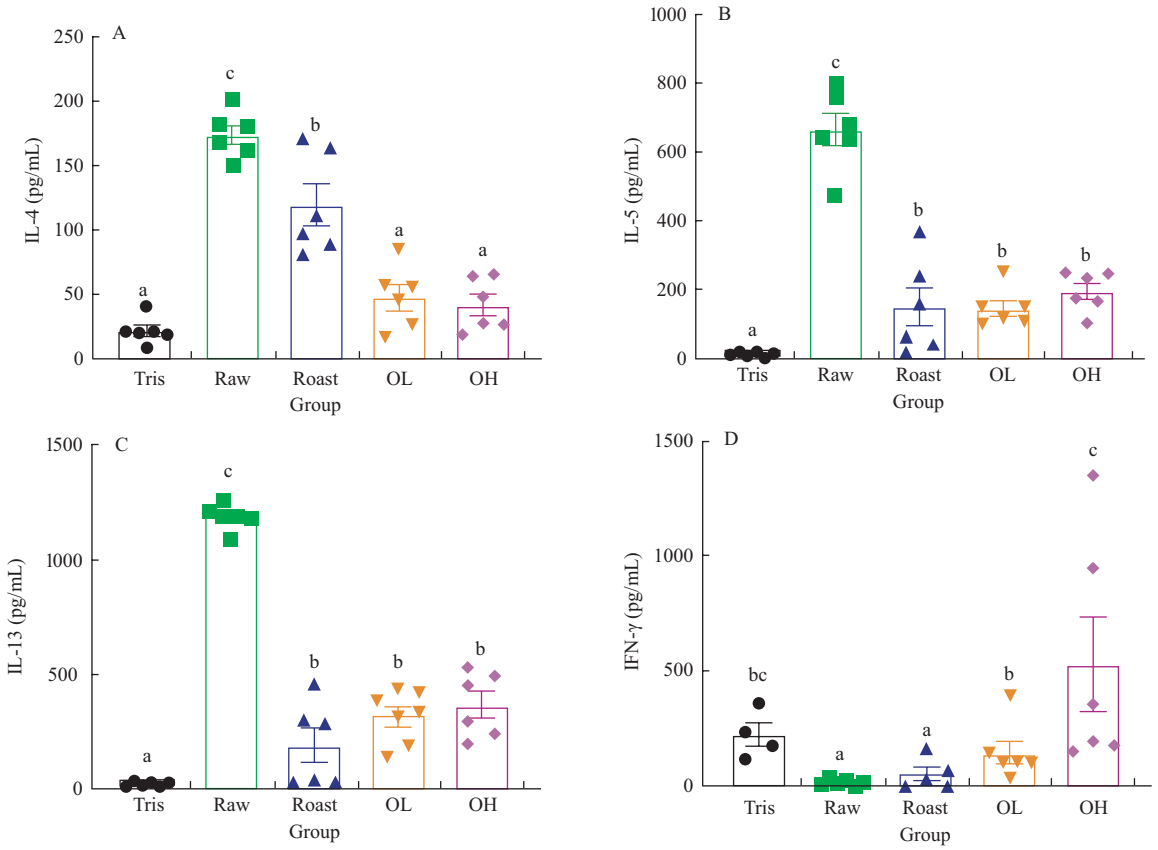

Fig. 4 Cytokine concentrations (pg/mL) were measured in the supernatant of *in vivo*-stimulated splenocytes with corresponding samples. (A) IL-4, (B) IL-5, (C) IL-13, and (D) IFN-γ. Different letters indicate significant differences ($P < 0.05$).

(Kyoto Encyclopedia of Genes and Genomes (KEGG), http://www.genome.jp/kegg/). These metabolites can be classified based on the pathways they are involved in or the functions they performed. Finally, pathway enrichment and topological analysis were performed. The Python package scipy.stats (https://docs.scipy.org/doc/scipy/) was exploited to identify statistically significantly enriched pathway using Fisher's exact test.

Fig. 5C shows the changes in the metabolic pathway in the mouse serum from the Raw group compared with that in Tris group, with 5 prominent pathways particularly listed. One prominent pathway is the tricarboxylic acid cycle (TCA cycle) pathway. Further analysis of the abundance of metabolites associated with the pathway revealed that four metabolites with significant changes (Fig. S1) revealed that the levels of citric acid and isocitric acid increased, and those of malic acid and oxoglutaric acid decreased.

Tryptophan metabolism was also prominent in the Raw group mice. The results of KEGG enrichment analysis indicated the downregulated overall expression of this pathway (Fig. S2B). Further analysis of the abundance of metabolites associated with the pathway revealed that the levels of seven metabolites, including *L*-tryptophan (Fig. 5E), xanthurenic acid (XA), quinoline-4,8-diol, tryptamine, *L*-arogenate, indoleacetaldehyde, and 4-(2-amino-3-hydroxyphenyl)-2,4-dioxobutanoic acid, all decreased significantly in the Raw group compared with the Tris group, and the related metabolites in the top 50 were labeled in red in the heat map (Fig. 5D).

Arginine and proline metabolism, lysine degradation, and arginine biosynthesis pathways also differed significantly between the 2 groups, and downregulated expression levels were observed in the Raw group (Fig. S2B). The involved partial metabolites include *L*-proline, *L*-lysine, and citrulline (Figs. 5F-H).

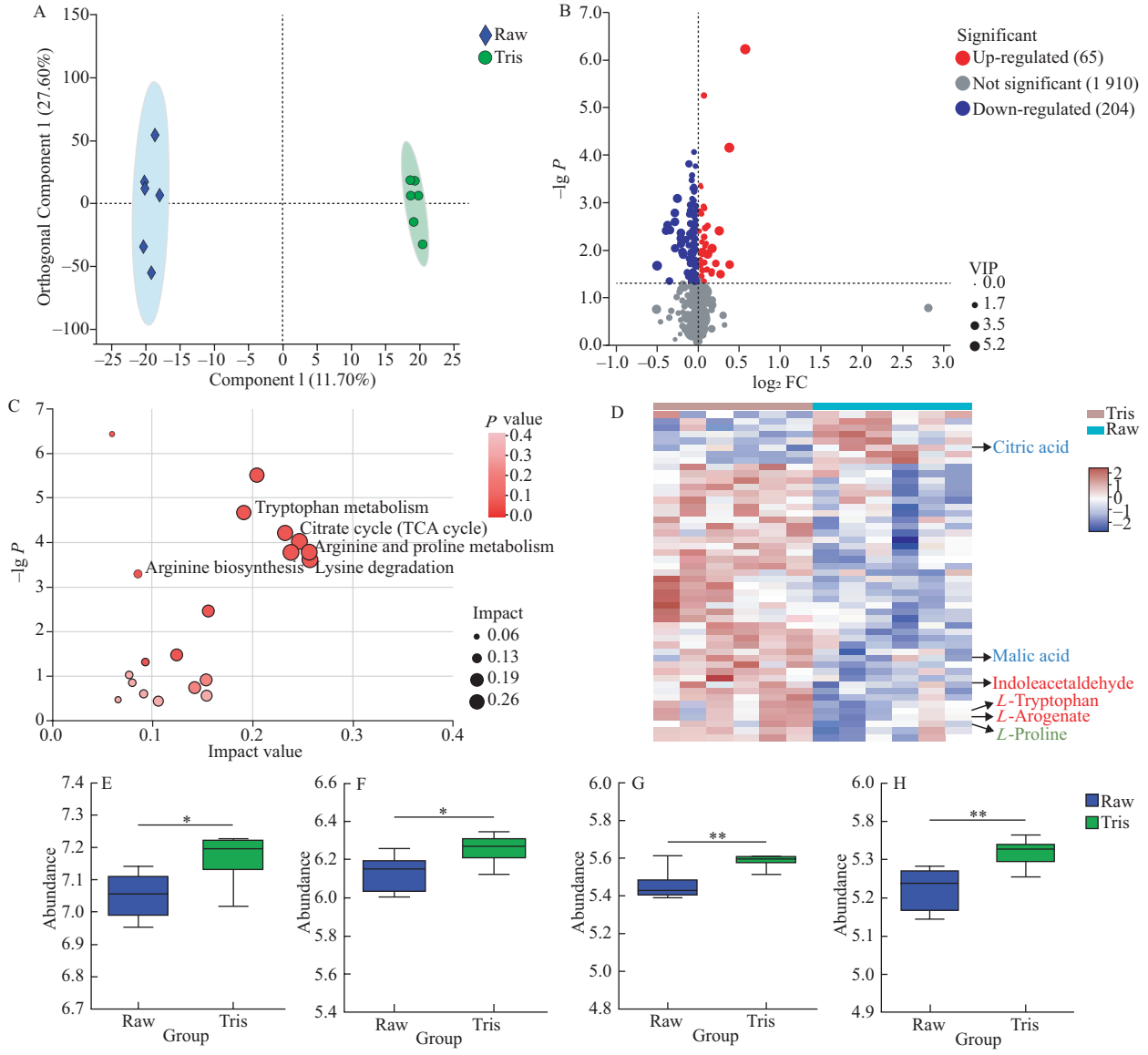

Fig. 5 Altered serum metabolite profile in mice in the Raw group. (A) OPLS-DA, (B) volcano plot and (C) KEGG topology analysis of all serum metabolites detected between the Raw and Tris groups ($n = 6$). (D) Heat map of all differentially expressed metabolites in the Raw and Tris groups (Top 50). Metabolites related to tryptophan metabolism, TCA cycle, and arginine and proline metabolism labeled in red, blue, and green, respectively. (E–H) significantly different abundances of *L*-tryptophan, *L*-proline, *L*-lysine, and citrulline between the Raw and Tris groups. Significant differences ($P < 0.05$) and very significant differences ($P < 0.01$) between groups were labeled as * and **, respectively.

3.6 Alterations in mouse serum metabolite profiles induced by roasted peanut proteins

Comparing profiles of serum metabolites from mice in the Roast, OL, and OH groups with those of Tris group (Fig. S2), the most prominent pathway was glycerophospholipid metabolism, which was downregulated in the Roast and OL groups and upregulated in the OH group.

Compared with Raw mice, when the mice were treated using the protein components of roasted peanut, the glycerophospholipid metabolism pathway in the serum has greatly affected (Figs. 6B-D). Metabolites involved in glycerophospholipid metabolism contained the mix of up- and down-regulated individual compounds (Fig. 6E), the global change of this pathway in 3 groups was upregulated after KEGG enrichment analysis (Fig. S3). In addition, other different pathways with significant changes in groups related to roasted peanut, such as the TCA cycle pathway, alanine, aspartate and glutamate metabolism pathway showed up-regulated trends in the Roast group, sphingolipid and tryptophan metabolism exhibited down-regulated trends in the OL group, and some other pathways presented up-regulated trends in the OH group (Fig. S3).

3.7 Different changes in the serum metabolites profiles induced by various protein components of roasted peanut

To analyze the differences in the metabolite profiles of mice challenged using various protein components from roasted peanut, we performed topological and abundance analyses of pathways (Fig. 7).

Compared with the total protein from roast peanut, OH induced significantly altered the expression of metabolites related to glycerophospholipid metabolism, lysine degradation, TCA cycle, and butanoate metabolism pathways, and OL induced significant alteration in the expressions of metabolites related to tyrosine, tryptophan, and glycerophospholipid metabolism (Figs. 7A-B). For glycerophospholipid metabolism pathway, the OH group showed an upregulated trend compared with the Roast and OL groups (Fig. 7C).

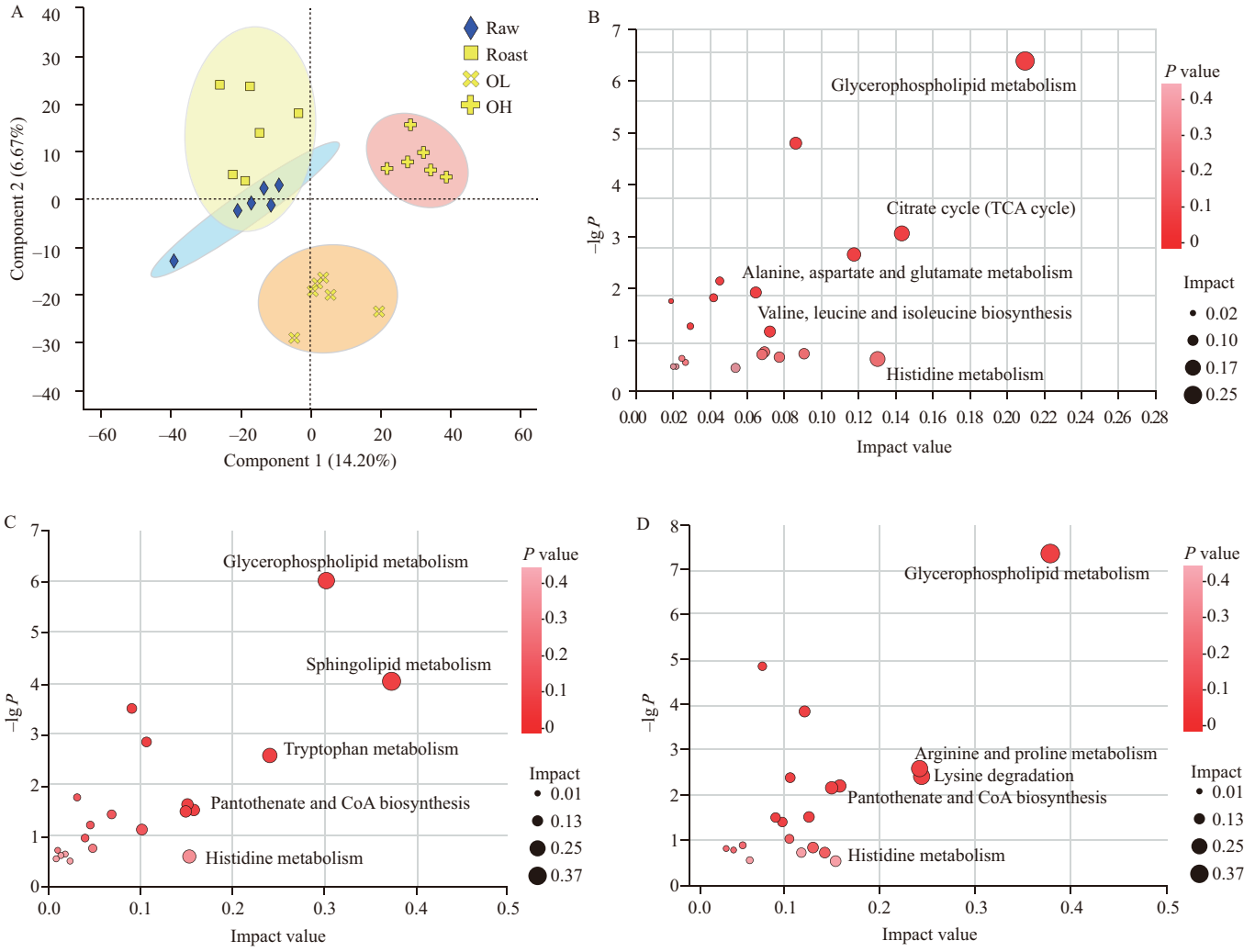

Fig. 6 Roast resulted in different serum metabolite profiles compared with Raw in mice. (A) PLS-DA plots among groups. (B-D) KEGG topological analysis of serum metabolites in mice challenged by protein components from roasted peanut compared with raw peanut. (B) Roast *vs*. Raw; (C) OL *vs*. Raw; (D) OH *vs*. Raw. (E) Heat map of metabolites related to glycerophospholipid metabolism pathway among different groups.

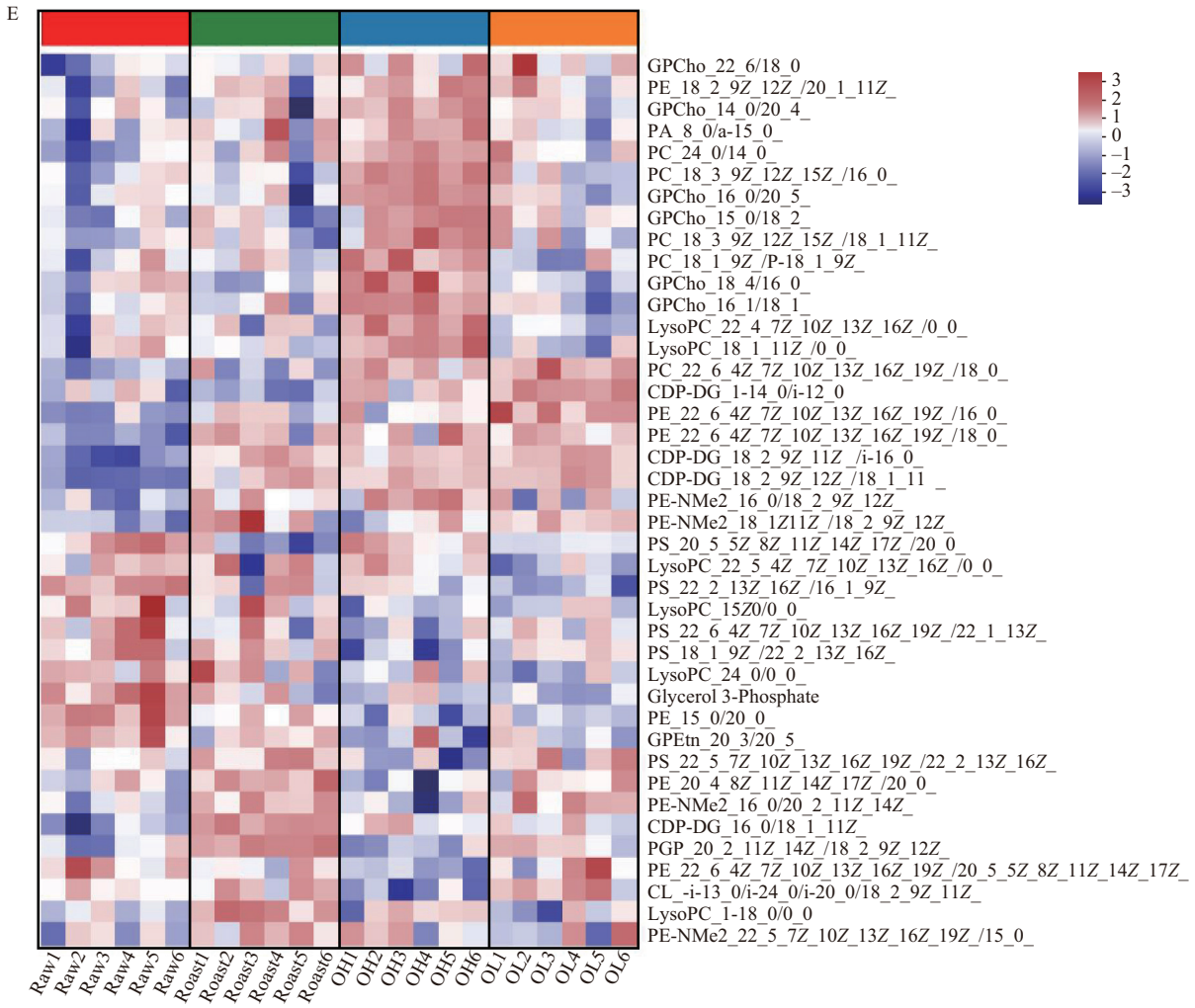

Fig. 6 (*Continued*)

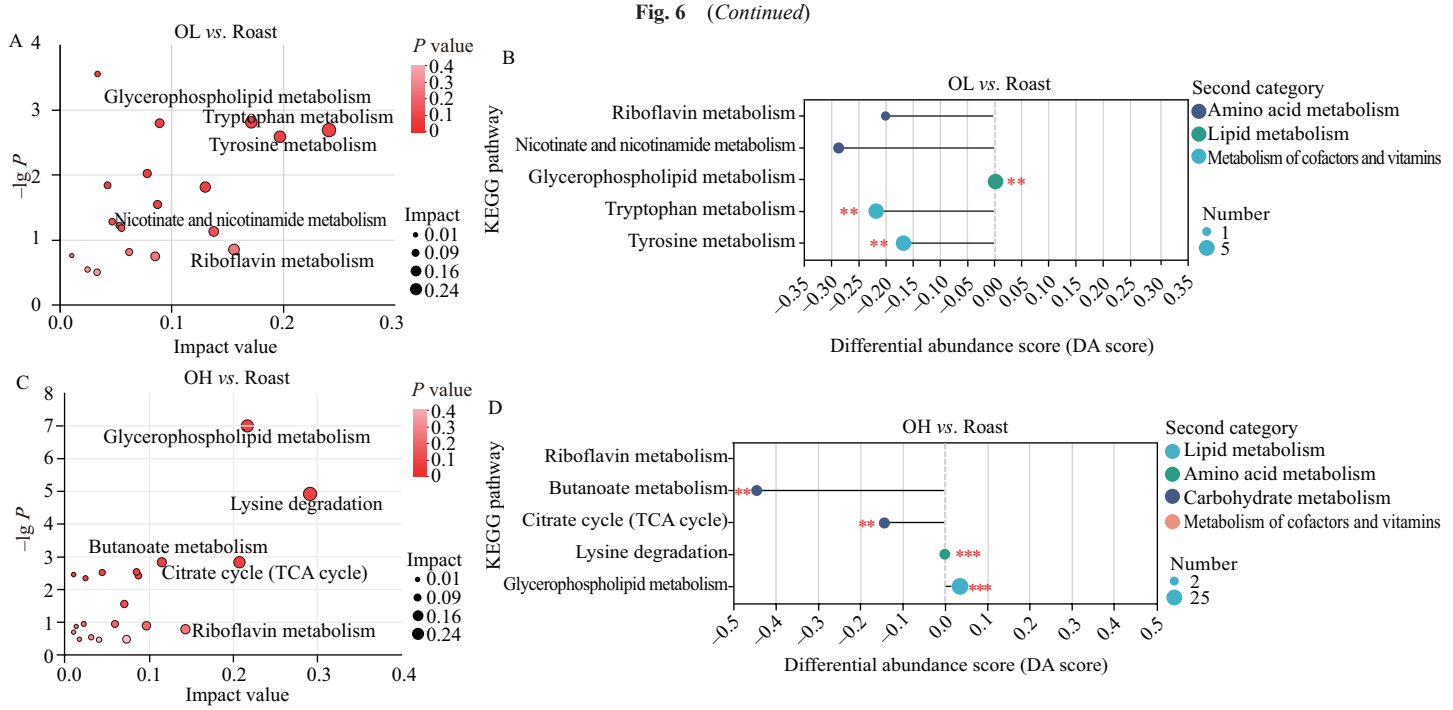

Fig. 7 OH and OL resulted in different serum metabolite profiles compared with the total Roast in mice. (A, C, and E) KEGG topological analysis of serum metabolites. (B, D, and F) KEGG enrichment analysis of serum metabolites. (A-B) OL *vs*. Roast; (C-D) OH *vs*. Roast; (E-F) OH *vs*. OL. $^{**}P < 0.01$, $^{***}P < 0.001$.

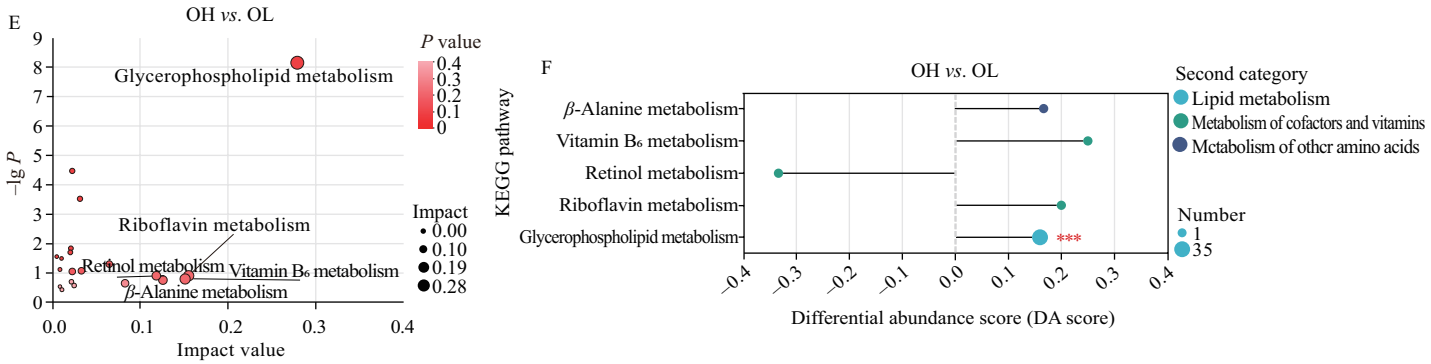

Fig. 7 (*Continued*)

4. Discussion

To discovery the effect of PA induced *via* raw and thermal processed peanuts on serum metabolism, we focused on Raw, Roast, OL and OH in this study. Peanuts were roasted at 170 °C for 12 min for pleasant flavor and better color (L^* of 57.83). The OL and OH separated in this work for the first time. Protein pattern of OL contained all allergen monomers. Protein pattern of OH mainly consisted of bands over 130 kDa and a small amount of proteins under 100 kDa. During protein elution, the eluent with 0.3 mol/L NaCl was sufficient to resolve all allergen monomers in the samples[21]; in the sample preparation for SDS-PAGE, the denaturation treatment with SDS caused breakage of noncovalent bonds between proteins; mass spectrometry data suggest that the bands around 43 and 60 kDa contained aggregation of allergen monomers or fragments[20]. These findings indicated that the low-molecular-weight bands in OH may include allergen monomers that formed noncovalent aggregations in Roast or covalently cross-linked allergen fragments, both of which are protein aggregations. Therefore, the protein components from Roast were successfully obtained.

PA can be marked by the expansion and degranulation of Th2 cells[32]; allergic symptoms and the levels of PP-sIgE and mMCP-1 can directly reflect the degree of allergic response in mice[33]. In this study, BALB/c mice challenged with the total protein from raw peanut (Raw group) exhibited noticeable allergic symptoms and significant elevation in the levels of PP-sIgE and mMCP-1. Meanwhile, the percentage of Th2 cells that differentiated in the splenocytes and released levels of cytokines related to Th2 cells increased significantly compared with those in the Tris group. These results illustrate that the mice in the Raw group had a typical Th2-type allergic reaction.

Raw peanut changed the serum metabolic profiles of mice. A previous study proved changes in serum metabolites in patients with certain food allergy[12], but they were not specific for PA. In this study, we performed serum untargeted metabolomics on mice from the PA group to gain insights into the underlying mechanisms involved in PA. Results reveal a distinctly metabolic signature associated with PA, characterized by notable alteration in carbohydrate metabolism and amino acid metabolic pathways. In detail, this finding was manifested as downregulated lysine degradation, arginine and proline metabolism, tryptophan metabolism, arginine biosynthesis pathways and disturbance of the TCA cycle pathway in peanut-allergic mice.

The TCA cycle involves cell metabolism and can generate citric acid initially. Then, citric acid can be converted into isocitric acid. In the secondary step, isocitrate is converted into oxoglutaric acid and subsequently into another four-carbon succinyl-ConA with the generation of nicotinamide adenine dinucleotide molecules. Next, succinyl-ConA converts into succinate, which is related to the generation of adenosine triphosphate. Succinate can also be oxidized to generate fumarate, the latter is converted into malate and further into oxaloacetate to continue the TCA cycle[34]. Intermediates in the TCA cycle play roles in immunity[35]; the level of citric acid changed significantly in peanut-allergic mice after 10 min post-challenge[36]. Our study, the levels of citric acid and isocitric acid were higher, and those of oxoglutaric acid and malic acid were lower in the mice in the Raw group than in the Tris group, which indicates that PA may disrupt the TCA cycle pathway.

Tryptophan is a kind of essential amino acid, its metabolism pathway includes direct transformation by gut microbiota into indole metabolites, 5-hudroxytryptamine pathway, and the kynurenine pathway in both immune and epithelial cells, which can produce metabolites for the regulation of immune response[12,37]. Tryptamine is a metabolite that originate from microbial metabolism pathway of tryptophan[38], with a significant antioxidant activity due to the effective roles in scavenging reactive radicals through its aminoindole structure[39]. Tryptamine plays an important role in the inhibition of gut inflammation and maintenance of intestinal balance[40]. the phenolic acid components make XA is one of the metabolites with the best free-radical scavengers in kynurenine pathway[41]. Compounds with antioxidant capability may attenuate allergic inflammatory responses in mouse models[42]. Notably, our study emphasized the downregulation of tryptophan metabolites in the serum of allergic mice. Specifically, compared with those in the Tris group, mice in the Raw group exhibited significantly reduced levels of tryptamine, XA, *L*-tryptophan and indoleacetaldehyde. The downregulation of tryptophan metabolites has been also revealed in an ovalbumin-sensitized mice model[43]. Mice in the Raw group showed changes in arginine and proline metabolism, which also was a enriched KEGG pathway in milk allergy patients[44].

Roast changed the capability of peanut protein to induce Th2-type allergic reaction. After the injection of protein from roasted peanut, the mice exhibited a lower anaphylactic response than those treated by raw peanut protein, especially in terms of cellular immunity. Specifically, the percentage of $CD4^+GATA3^+$ Th2 cells that differentiated in splenocytes and the levels of cytokines related to Th2 cells decreased significantly in mice challenged with proteins from roasted peanut (Roast, OL, and OH groups) compared with those challenged with Raw. These results may be due to the altered structure of peanut protein after roasting[45]. The OL group with various

allergen monomers exhibited different changes in structures[21]. Roast modified the overall and local structure of allergens[21] and the potential sensitization and internalization of allergen monomers, such as Ara h 3[46] and Ara h 2[47], in the initial phase of allergy, to change allergenicity. In OH, the allergen aggregation formed *via* covalent and noncovalent bonds among allergens, with or without intramolecular crosslinking in allergen molecule, and together with changes in the structures of IgE epitopes[22]. Moreover, OH can be formed by fragments of allergens, and the breakage of allergens may change or destroy IgE epitopes[48], which will influence allergenicity.

Roasted peanut induced various changes in the serum metabolite profiles of mice compared with raw peanut. Glycerophospholipid metabolism was the most affected pathway in roast-treated mice, followed by tryptophan metabolism. Glycerophospholiqids had been experimentally shown to be involved in shrimp allergy[49], and decreased levels of glycerophospholipid compounds had been observed in house dust mite-sensitized mice[50]. Moreover, glycerophospholipid metabolism is the most significantly altered pathway in a mouse model of allergic asthma[51]. In this study, glycerophospholipid metabolism was downregulated in the Raw group compared with Tris group, although it is not listed in the top 5 significantly relevant pathways (data not shown). Meanwhile, glycerophospholipid metabolism in the Roast group was down-regulated compared with that in the Tris group but was up-regulated compared with the Raw group, which showed opposite trends in changes in the Th2-type immune response and allergic symptoms. This phenomenon also occurred in the OL group. These results suggest that glycerophospholipid metabolism may be another important pathway related to PA, and it may be involved in the severity of allergic reactions, which need to be further confirmed by more experiments in the future. The differences in serum metabolic profiles caused by various proteins implied that the processing form of allergen and severity of allergy should be considered in the analysis of serum biomarkers.

Various fractions of roasted peanut protein affected the metabolite profiles of serum differently. Compared with the Raw group, glycerophospholipid metabolism was the most affected pathway in mice in the 3 groups related to roasted peanut. Beyond that, other pathways were influenced to different degrees. Compared with roasted peanuts, OL induced greater influence on tryptophan and tyrosine metabolism, and OH caused the opposite trend in glycerophospholipid metabolism.

The causes of different serum metabolic profiles induced by samples are complex. On the one hand, serum metabolomic profiling is associated with the severity of food allergy. The metabolomic profiling from clinical samples indicated that food allergy was uniquely associated with decrease in sphingolipid and other lipid metabolites, and children with severe food allergy with alteration in tryptophan metabolism[12]. In our study, mice treated by different proteins all showed decreased glycerophospholipid metabolism, and those in the Raw group exhibited the most significant robust Th2-immunoreaction and a decrease in metabolites of tryptophan. On the other hand, serum metabolomic profiling may be influence by chemical modification of protein components. Roast, OL, and OH with different structural features and allergen monomers in OL not only showed structural changes but also more modifications on the side chains, such as methyl and oxidation modifications, than Raw[20]. Allergens in OH not only participated in crosslinking but also revealed numerous chemical modifications related to advanced glycosylation end products[22]. The effects of protein posttranslational modification on enzyme function, receptor activation, cell interactions, cell metabolism, and signaling pathways have been discussed[12], which indicates that chemical modification in allergens caused by thermal processing may be additional regulatory factor in food allergic disease.

5. Conclusion

Our study established a peanut allergic mouse model to present the metabolomic signatures in mice challenged with different protein components. Results reveal that PA induced using raw peanuts brought massive changes in amino acid metabolism, including tryptophan metabolism, and induced disruption of the TCA cycle pathway. Roast treatment decreased the Th2 polarization of immune cells in mice induced by peanut protein, and the main pathways affected by roasted peanut were glycerophospholipid and tryptophan metabolism. Compared with roasted peanuts, OL induced a greater influence on tryptophan and tyrosine metabolism, and OH caused the opposite trend in glycerophospholipid metabolism. The differences in serum metabolic profiles caused by different proteins implied that the processing form of allergen that patients are sensitized to and the severity of allergy should be considered in the analysis of biomarkers. These results provide new insights into the fields of potential metabolomic marker for PA may aid researchers in developing strategies for clinical intervention in peanut allergic patients.

Conflicts of interest

All the authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2024.9250447.

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